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Antihypertensive Effect of Amaranth Hydrolysate Is Comparable to the Effect of Low-Intensity Physical Activity

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Received: 18 July 2020; Accepted: 13 August 2020; Published: 17 August 2020

Abstract: *Background and objectives:* Both antihypertensive peptide intake and physical activity help to control blood pressure. Our aim was to evaluate the impact of consuming amaranth antihypertensive peptides on systolic blood pressure (SBP) in normotensive rats and the magnitude and relevance of the peptide-induced antihypertensive effect in spontaneously hypertensive rats (SHR). *Materials and Methods:* Treatments (alcalase-generated amaranth protein hydrolysate, captopril, or water) were given by gavage and the SBP measured by the tail-cuff method. Physical activity was performed five days/week (for twenty weeks). *Results:* The normotensive rats' SBP (mmHg, average/group) remained unaffected after amaranth antihypertensive peptide supplementation (121.8) ($p > 0.05$ vs controls). In SHR, the SBP was lowered by 24.6 (sedentary/supplemented at two weeks), 42.0 (sedentary/supplemented at eight weeks), and 31.5 (exercised/non-supplemented at eight weeks) ($p < 0.05$ vs sedentary/non-supplemented). The combination of supplementation and physical activity lowered the SBP by 36.2 and 42.7 (supplemented/exercised at two weeks and eight weeks, respectively) ($p < 0.05$ vs sedentary/non-supplemented), but it did not have additional antihypertensive benefits ($p > 0.05$ vs sedentary/supplemented at eight weeks or exercised/non-supplemented at eight weeks). *Conclusions:* Amaranth antihypertensive peptide supplementation has no impact on SBP in normotensive rats. This supplementation develops sustained antihypertensive benefits in SHR, which are similar to the antihypertensive effect developed after eight- or twenty-week low-intensity physical activity. These findings have implications for developing safe and effective peptide-based functional foods.

Keywords: amaranth hydrolysate; physical activity; ACE-1; hydrolysis

1. Introduction

Hypertension is defined as high blood pressure and is a risk factor for cardiovascular diseases [1]. Inhibiting specific components of the renin–angiotensin–aldosterone system is a common strategy for controlling blood pressure. Compromising the catalysis of the proteases renin and angiotensin-converting enzyme 1 (ACE-1) reduces the formation of angiotensin, diminishing vasoconstriction, aldosterone secretion, and sodium and fluid retention [2]. Some food-derived peptides can inhibit ACE-1 activity and, therefore, could help to control blood pressure. Commonly, non-human proteases are utilized to generate hydrolysates with potent antihypertensive peptides [3,4]. Recent findings demonstrate that an optimized amaranth hydrolysate generated with food grade alcalase (E.C. 3.4.21.62) inhibits ACE-1 in vitro and lowers the systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) [4–6]. This antihypertensive effect is developed in the first few hours after the amaranth hydrolysate intake [4–6]. However, uncertainty remains regarding the relevance of such an antihypertensive effect beyond a few hours, the impact of consuming amaranth hydrolysate on blood pressure in normotensive cases, and the magnitude of the antihypertensive effect compared to lifestyle interventions such as physical activity. Increasing physical activity helps to prevent and control blood pressure [7–9], in part by promoting the exercise-induced attenuation of sympathetic vasoconstriction [10–12]. In fact, a physical activity program of at least 30 min five times per week is an inexpensive and effective intervention that helps to control blood pressure [13]. Thus, the aim of the present study was to evaluate the impact of consuming amaranth antihypertensive peptides on SBP in normotensive and hypertensive cases, as well as the relevance of their regular intake and the magnitude of the peptide-induced antihypertensive benefits, taking as a reference a low-intensity physical activity program.

2. Materials and Methods

2.1. Extraction and Concentration of Amaranth Protein

A commercially available amaranth protein isolate (COPRAM™) was used to obtain an amaranth protein concentrate. The isolate (~30% protein) was suspended in acetone (1:2 weight/volume), stirred vigorously, and centrifuged (6000× g, 30 min, 4 °C). The precipitate was collected and re-suspended in NaOH (62.5 mM), stirred (24 h, 4 °C), and centrifuged (6000× g, 20 min, 10 °C). The supernatant was collected, and the pH was adjusted to 5.0 to precipitate the proteins. The suspension was centrifuged (6000× g, 20 min, 10 °C), the precipitate collected and re-suspended in water, and the pH adjusted to 7.0 [14]. Finally, the protein concentrate was freeze-dried and stored at room temperature. The total protein content was evaluated following the Association of Official Analytical Chemists (AOAC) method 979.09 [15].

2.2. Hydrolysis Reaction

The amaranth protein concentrate was hydrolyzed as previously described [4]. Briefly, the protein concentrate was re-suspended in potassium phosphate buffer (75 mM, pH = 7.01) and incubated for 1 h at 52 °C before the addition of alcalase (0.04 mU/mg of protein). The alcalase-based hydrolysis reaction was carried out at 52 °C for 6.16 h and stopped by heating (85 °C, 10 min). The hydrolysate was freeze-dried and stored at room temperature until its use.

2.3. Animals and Ethical Issues

Male SHR and *Wistar Kyoto* normotensive rats (8 weeks old, 300–350 g body weight) were provided by the Cell Physiology Institute of the National Autonomous University of Mexico. The rats were placed in plastic cages with stainless steel lids and maintained at 28 °C with 12:12 h light-dark

cycles. Food (Rodent Lab Chow 5001, Ralston Purina Inc., St. Louis, MO, USA) and water were available ad libitum. An Ethics Review Board of the Autonomous University of Sinaloa approved the study protocol (CE-UACNYG-2015-SEP-001).

2.4. Effect of the Supplementation with Amaranth Protein Hydrolysate on SBP in Normotensive Rats

Normotensive *Wistar Kyoto* rats (n = 24) were randomly allocated into three groups: supplemented with amaranth hydrolysate (1.2 g/kg body weight) [4], a captopril control group (solubilized in water; 25 mg/kg body weight) [16–18], and water only (non-supplemented). An additional group of supplemented SHR (n = 8) was used for comparison purposes (Figure 1A). Orogastric feeding tubes were used for treatment administration (18GA_75 mm, Instech Laboratories, Inc., Plymouth meeting, PA, USA). SBP measurements were carried out by the tail-cuff method (CODA tail cuff, Kent Scientific, Torrington, CT, USA). The SBP evaluations were carried out before (time 0) and after supplementation at 1 h intervals for 7 h (Figure 1A).

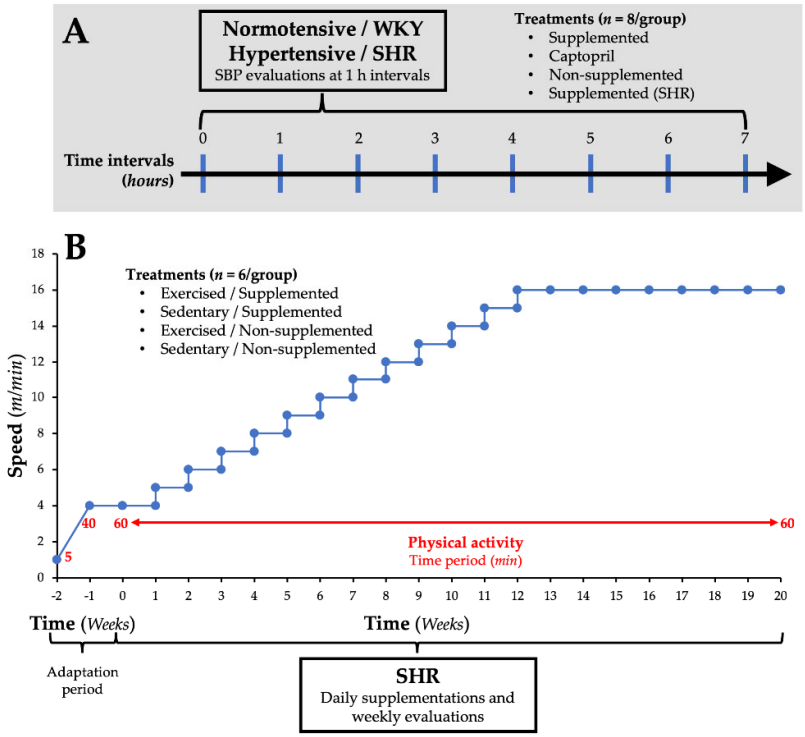

Figure 1. Scheme of interventions. Part (**A**): Evaluations of SBP in normotensive (WKY) and hypertensive (SHR) rats. WKY were randomly allocated into three groups, and an additional group of SHR was evaluated. All treatments were administered by gavage. Part (**B**): Evaluations of SBP in SHR. SHR were randomly allocated into four groups. Supplementations were carried out by gavage. Physical activity time period and intensity were gradually increased from 5 to 60 min and from 1 to 16 m/min, respectively. SBP: systolic blood pressure. WKY: *Wistar Kyoto* rats; SHR: spontaneously hypertensive rats.

2.5. Effect of the Supplementation with Amaranth Protein Hydrolysate and the Implementation of a Low-Intensity Physical Activity Program on SBP in SHR

SHR (*n* = 24) were randomly allocated into four groups: exercised and supplemented with amaranth hydrolysate (exercised/supplemented (1.2 g/kg of body weight)), sedentary and supplemented (sedentary/supplemented), and exercised or sedentary, but non-supplemented (exercised/non-supplemented and sedentary/non-supplemented, respectively). All the rats underwent a two-week adaptation period before starting any intervention. Figure 1B shows the

scheme of the interventions. Animals were supplemented daily and the SBP measurements carried out weekly before the supplementation of the day was performed (CODA tail cuff, Kent Scientific, Torrington, CT, USA). The physical activity was performed in a motorized treadmill, increasing the speed progressively in the first week of the adaptation period (rat forced exercise walking wheel system; 80805A; Lafayette Instruments, Lafayette, IN, USA) (Figure 1B). The purpose of the adaptation period was to improve the rats' resistance in order to keep them on physical activity for 60 min (4 m/min). After this period, the physical activity was sustained for 60 min at all stages of the interventions, but the speed was increased weekly by 1 m/min until a maximum speed of 16 m/min was reached (Figure 1B) [19,20].

2.6. Statistical Analysis

The Shapiro–Wilk normality test was used to assess data distribution. Comparisons were carried out by analysis of variance and Tukey's test or Kruskal–Wallis test. The results are presented as means with standard deviations or medians with interquartile range. The GraphPad Prism 7.0 software was used. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Lack of Antihypertensive Effect of Amaranth Protein Hydrolysate in Normotensive Rats

The SBP lowered after supplementation with amaranth protein hydrolysate in SHR (Figure 2). This positive reduction in SBP started after three hours of supplementation (13.5 mmHg, average; p < 0.05 vs normotensive groups), but the strongest antihypertensive effect was developed after six and seven hours (52.0 and 48.1 mmHg on average, respectively). The magnitude of the amaranth peptide-induced antihypertensive effect was remarkable in SHR; in fact, the SBP lowered to units very close to the ones documented in normotensive rats (Figure 2). Neither amaranth protein hydrolysate nor captopril lowered the SBP in normotensive rats (p > 0.05 vs non-supplemented (water only)), and there was not even any trend to lower it (Figure 2).

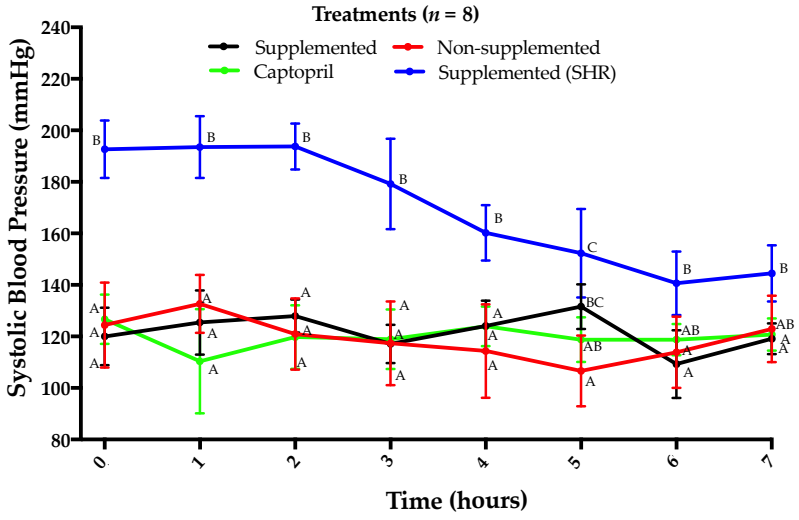

Figure 2. Evaluation of SBP in normotensive WKY and SHR. WKY (n = 24) were randomly allocated into three groups: supplemented (amaranth hydrolysate, 1.2 g/kg body weight), captopril (25 mg/kg body weight), and non-supplemented (water only; 1.5 mL). An additional group of supplemented SHR (n = 8) was evaluated (1.2 g/kg body weight). Different letters (A, B, or C) indicate significant differences among groups at the same week. The data were analyzed by analysis of variance and Tukey's test. The results are shown as means with standard deviations.

3.2. *Supplementation with Amaranth Hydrolysate and Implementation of a Low-Intensity Physical Activity Program Have Similar Antihypertensive Effects*

By week two of the interventions, both the exercised/supplemented group and the sedentary/supplemented group had lowered their SBP by 36.2 and 24.6 mmHg, on average, respectively ($p < 0.05$ vs sedentary/non-supplemented and exercised/non-supplemented) (Figure 3). This significant positive reduction in SBP was developed at week one in the exercised/supplemented group (38.1 mmHg, average). From week two until the end of the interventions, the antihypertensive effects were sustained in both the exercised/supplemented group and the sedentary/supplemented group ($p < 0.05$ vs sedentary/non-supplemented). A slight increase in SBP was observed in the sedentary/supplemented group from week eleven until week seventeen. This indicates that absolute SBP can be lowered, but SBP variations were not adequately controlled. A similar event was observed for the exercised/supplemented group (Figure 3). The lowest positive reduction in SBP was recorded at week five in the sedentary/supplemented group (49.2 mmHg, average) and at week sixteen in the exercised/supplemented group (55.2 mmHg, average) (Figure 3).

The exercised/non-supplemented group started lowering their SBP at week seven of the intervention (8.5 mmHg, average; $p > 0.05$ vs sedentary/non-supplemented). At week eight, the SBP recorded showed an additional positive reduction (31.5 mmHg, average; $p < 0.05$ vs sedentary/non-supplemented, $p > 0.05$ vs exercised/supplemented and sedentary/supplemented) (Figure 3). The positive reduction in SBP was sustained until the end of the intervention ($p < 0.05$ vs sedentary/non-supplemented, $p > 0.05$ vs exercised/supplemented and sedentary/supplemented). The lowest positive reduction in SBP was recorded at week nineteen (52.0 mmHg, average) (Figure 3).

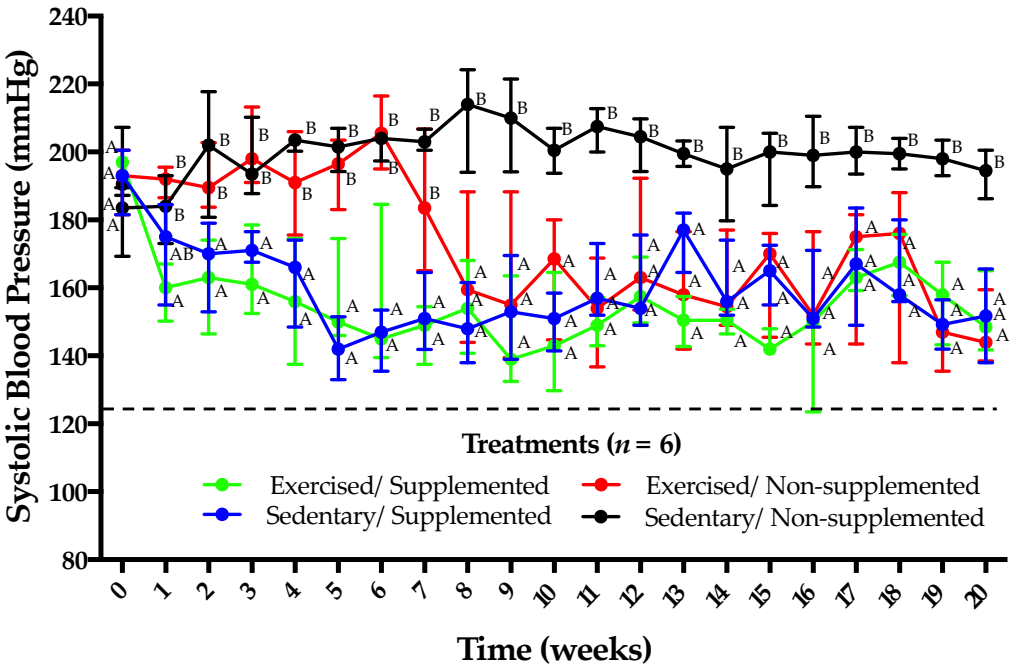

Figure 3. Evaluation of SBP in SHR. SHR (n = 24) were randomly allocated into four groups: exercised/supplemented, sedentary/supplemented, exercised/non-supplemented, and sedentary/non-supplemented. Supplementations consisted of amaranth hydrolysate (1.2 g/kg body weight). See Figure 1 for physical activity details. The dotted line represents the average SBP of eight aged-matched normotensive rats. Different letters (A, B, or C) indicate significant differences among groups at the same week. The data were analyzed by Kruskal–Wallis test. The results are shown as medians with interquartile range.

4. Discussion

Alcalase-generated amaranth peptides can lower the SBP in SHR through ACE-1 inhibition [4]. This enzyme inhibition hampers the formation of the potent vasoconstrictor angiotensin II and reduces the hydrolysis of the endothelium-dependent vasodilator bradykinin [21,22]. ACE-1 inhibitor drugs are widely used for controlling blood pressure mainly because they are low-cost, effective drugs and their potential side effects cause discomfort but are not life-threatening, with the exception of angioedema [23]. The alcalase-generated amaranth protein hydrolysate evaluated in the present study inhibits ACE-1 and can be used as a food-derived antihypertensive ingredient for developing functional foods [5,6]. These ACE-1-inhibiting foods could be used not only to help control hypertension but also other cardiovascular diseases, since ACE-1 inhibitors are useful for treating systolic and diastolic left ventricle dysfunction and acute coronary syndrome, among other conditions [24]. Ideally, antihypertensive functional foods should not trigger side effects neither in the target nor the healthy population. The rationale for the latter relies on the fact that in normotensive organisms that lack ACE-1 or have reduced serum levels of it, blood pressure could be unnecessarily lowered [25], and also that hypotension, a physiological side effect, could occur as a first-dose phenomenon after ACE-1 inhibitor administration [23,26]. The present study shows that at first dose, the oral administration of the alcalase-generated amaranth protein hydrolysate has an effect similar to captopril in normotensive rats and SHR [4,27–29]. This implies a lack of any effect on SBP in normotensive rats, but a significant antihypertensive effect in SHR. Other researchers have reported similar findings using milk-derived peptides [30,31]. For some [32], the use of ACE-1 inhibitor drugs should be replaced with angiotensin receptor blockers to treat hypertension. This observation is based on data supporting that the rate of cough (9.9% vs 3.2%) and the incidence of angioedema (0.3% vs 0.13%) are significantly greater in patients treated with ACE-1-1 inhibitor drugs than in those treated with angiotensin receptor blockers. Overall, our findings should be taken as the first proof of concept about the safety of alcalase-generated amaranth protein hydrolysate, which supports the development of functional foods for controlling SBP or for use as an adjunct in lifestyle interventions in essential hypertension cases and potentially in other cardiovascular diseases.

The antihypertensive effect of alcalase-generated amaranth protein hydrolysate can occur as fast as three hours after its administration by gavage, as shown in the present and other studies [4]. Additionally, the present study shows that this antihypertensive effect can be sustained for weeks or months as long as the hydrolysate is consumed in specific doses once a day. In long-term use of ACE-1 inhibitors, the inhibition of this enzyme in tissues other than blood becomes more relevant since angiotensin II can be produced through alternative pathways [24,33], e.g., ACE-independent angiotensin II production by the serine protease chymase [33,34]. In SHR, there is a trend to produce high levels of angiotensin II due to angiotensinogen being overexpressed at the intrarenal level [34–36]. Current evidence supports that alcalase-generated amaranth peptides that enter into the blood stream after protein hydrolysate consumption can inhibit ACE-1 in plasma [4], but it does not directly support that food-derived peptides can inhibit ACE-1 in the kidneys, lungs, or tissues other than blood. The fact that amaranth peptides can develop a sustained antihypertensive effect suggests that these food-derived peptides can act in tissues other than blood. Certainly, the mechanism underlying ACE-1 inhibition among different compounds, including peptides, is not expected to largely differ but rather the main targeted organ and pharmacokinetics will determine the clinical effect [24]. In line with previous studies [22,37], the prevention of bradykinin type 2 receptor desensitization and improvement of the circulating levels of bradykinin can be important for the sustained antihypertensive effect promoted by the regular consumption of hydrolysate in addition to ACE-1 inhibition. In light of current knowledge, the results open specific questions about the mechanisms underlying the antihypertensive effect of food-derived peptides, and with certainty show that the use of these peptides for developing antihypertensive functional foods for long-term use is feasible.

It is widely accepted that physical activity is helpful in lifestyle interventions for treating patients with hypertension [38,39]. Compelling evidence suggests that exercise stimuli increase the endothelial function and that this biological event increases the activity of endothelial nitric oxide synthases [40,41]. As a mediator of functional sympatholysis, nitric oxide promotes the exercise-

induced attenuation of sympathetic vasoconstriction [11,12]. As stated by others [19,42], our results show that a low-intensity aerobic exercise routine can lower the SBP in SHR and that the antihypertensive effect can be sustained as the physical activity is conducted. The SBP started lowering at week 7 of physical activity. This delay in the exercise-induced antihypertensive effect has been reported by others [19] and fits with the hypothesis that the exercise-induced antihypertensive benefits may involve the summation over time of "sub-acute effects of exercise" (individual sessions of physical activity) until a "chronic blood pressure response to exercise" is achieved [43,44] and/or other physiological changes occur, e.g., improvement of kidney ultrastructure in some cases [19]. Due to the delay in achieving the expected significant antihypertensive effect of the physical activity, the use of ACE-1 inhibitors seems to be advantageous in the first few weeks of this lifestyle intervention for treating hypertension. In this context, the alcalase-generated amaranth protein hydrolysate can significatively lower the SBP a few hours after its consumption, and the antihypertensive effect can be sustained for weeks as long as it is regularly consumed. Notably, the antihypertensive effect of hydrolysate is similar in magnitude to the effect developed after eight- or twenty-week low-intensity physical activity, but without the additional benefits of an aerobic exercise routine, e.g., muscle regeneration and improvement of metabolic disorders, among other benefits [20]. The present study also shows that the combination of low-intensity physical activity and supplementation with amaranth hydrolysate does not have additional antihypertensive benefits. In this context, we should acknowledge that our study has some limitations. Firstly, we evaluate only one low-intensity physical activity protocol, which has proven to be effective for lowering the SBP in SHR [19], but we are uncertain if this is the best physical activity protocol for developing antihypertensive effects. Secondly, although we have proven that alcalase-generated amaranth protein hydrolysate can be used as an ingredient for developing effective antihypertensive functional foods [5,6], the hydrolysate itself rather than a food was used as the source of antihypertensive peptides and peptide identification analysis was not performed. Finally, there is the possibility that larger groups of animals would emphasize the antihypertensive effects observed. Despite these limitations, the study highlights that there is a reduced risk of symptomatic hypotension after the consumption of the antihypertensive amaranth hydrolysate, and that the magnitude and relevance of the antihypertensive effect developed (sustained positive reduction in SBP of at least 24 mmHg) is sufficient to significantly reduce the risk of coronary heart disease, stroke, heart failure, and mortality [45].

5. Conclusions

An alcalase-generated amaranth protein hydrolysate efficiently lowered the SBP in SHR while it had no effect in normotensive cases. The magnitude of this antihypertensive effect is comparable to the effect developed after eight- or twenty-week low-intensity physical activity for 60 min 5 times per week. The relevance of hydrolysate for use in the development of ACE-1 inhibitor functional foods relies on the fact that the antihypertensive benefits of its consumption can be sustained for months as long as it is consumed once a day. These findings have implications for the development of safe and effective antihypertensive functional foods using food-derived peptides as functional ingredients and serve as groundwork for designing future clinical trials.

Author Contributions: Conceptualization, F.C.-C. and N.O.; Formal analysis, F.C.-C., V.L.-T., F.I.C.-T., H.F.A.-G., J.A.H.-M., G.G.-O., G.I.R.-T., and N.O.; Funding acquisition, F.C.-C.; Investigation, V.L.-T., E.R.R.-B., G.I.R.-T., and N.O.; Methodology, P.Y.G.-A. and G.I.R.-T.; Project administration, F.C.-C.; Software, F.I.C.-T.; Supervision, N.O.; Visualization, P.Y.G.-A. and H.F.A.-G.; Writing—original draft, F.C.-C., G.I.R.-T., and N.O.; Writing—review and editing, F.C.-C., P.Y.G.-A., F.I.C.-T., E.R.R.-B., H.F.A.-G., J.A.H.-M., G.G.-O., and G.I.R.-T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Universidad Autónoma de Sinaloa, grant number PROFAPI2015/049.

Acknowledgments: The authors wish to thank CONACyT for the doctoral fellowships given to F.I.C.-T. and G.I.R.-T. The protein isolate (COPRAM™) was kindly donated by San Miguel de Proyectos Agropecuarios S.P.R. de R.S.

Conflicts of Interest: The authors declare no conflicts of interest.

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